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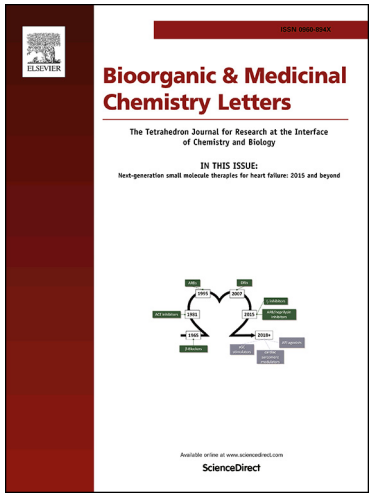

Biofilm inhibition and anti-*Candida* activity of a cationic lipo-benzamide molecule with twin-nonyl chain

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Biofilm inhibition and anti-*Candida* activity of a cationic lipo-benzamide molecule with twin-nonyl chain

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ABSTRACT

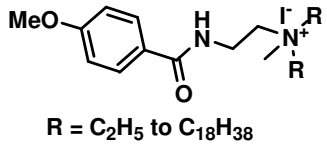
A series of cationic lipo-benzamide compounds with varying lengths of hydrocarbon chains (C2M-C18M) were evaluated for anti-Candida activity. Four compounds harbouring 8-11 hydrocarbon chains demonstrated concentration-dependent inhibition of fungal cell growth with Minimum Inhibitory Concentration (MIC) of ≤ 6.2 µg ml^{-1}. The most active compound (C9M) inhibited growth of both Candida albicans and non-albicans strains and is equally active against pairs of azole sensitive and resistant clinical isolates of C. albicans. Compound C9M also inhibited different stages of Candida biofilms. Scanning Electron Microscopy (SEM) of Candida cells after C9M treatment was also done and no significant cell lysis was observed. Hemolysis assay was performed and only 2.5% haemolysis was observed at MIC concentration.

Introduction

Fungal infections are one of the most pressing public health care concerns worldwide [1]. The human microbiota also consists of fungi that render several beneficial functions. In spite of tremendous health benefits, some microbiota can cause mild to severe infections, especially in conditions of immune-suppression [2]. *Candida albicans* is one such fungus which is a part of commensal microflora in humans but becomes invasive and lethal when the immune system of an individual is compromised [3]. *Candida* causes cutaneous, subcutaneous, mucosal (localized-*candidiasis*), as well as systemic infections (*Candidemia*) [4]. Candidemia or *Candidiasis* has been a leading type of nosocomial infection with high morbidity and mortality rates (20–40%) [5]. Conventional antifungal treatment for *Candidiasis* includes polyenes, azoles and recently launched echinocandins [6]. However, conventional treatment options have a narrow therapeutic index, poor bio-availability, severe side effects and the chance of emergence of resistant strains [7]. Therefore, the limitations associated with conventional treatment necessitate the exploration of new drug candidates that can get rid of drug-resistant and lethal systemic mycoses.

Quaternary ammonium compounds (QACs) have been known to be the most useful antiseptics and disinfectants [8]. The cationic agents supposedly react with the phospholipid components of the cytoplasmic membrane, thereby producing membrane distortion and a net positive charge on microbial cells [9]. The positive charge on microbial cells has often been correlated to their biocidal action. Some polymeric QACs have been shown to induce lysis of spheroplasts of *Serratia marcescens*, but not those of *C. albicans* [10]. The critical phenomenon determining the antifungal effect of cationic surfactants and lipids is not cell lysis but rather the reversal of cell surface charge from negative to positive that leads to membrane distortion [9]. QAC's fused to varying length of carbon chains are reported to have antimicrobial activities. It was reported that QAC's fused to 10-12 methylene linkers showed growth inhibitory effect against drug-resistant bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [11]. Moreover, molecules with a net positive charge were not only able to kill microorganisms in solution but also upon attachment or adsorption onto solid surfaces [12]. The organic monolayers containing quaternary ammonium groups have been shown to prevent deposition and growth of bacterial biofilm [13]. However,

the activity of such compounds on fungal biofilm formation and maturation remain elusive.



Scheme 1: Represents general structure of lipo-benzamide.

Our present study focuses primarily on the antifungal activity of hybrid quaternary ammonium compounds (QACs) previously synthesized[14] by our group and showed promising anticancer activity. Lipo-benzamide compounds fused with varying lengths of hydrocarbon chains C2M - C18M (Scheme 1) were evaluated for antifungal activity against *C. albicans.* The most active compound was tested for growth inhibition against azole-resistant clinical isolates of *C. albicans* as well as non-*albicans Candida* strains. The ability of such cationic lipo-benzamides to inhibit adhesion of microbial cells to polystyrene surfaces as well as on biofilm growth was further evaluated.

Table 1. Anti-*Candida* activity of cationic lipo-benzamide C9M. Fluconazole was used as standard anti-fungal agent.

Strain	MIC (μg ml^{-1})[a]	
	Fluconazole	**C9M**
Candida albicans ATCC90028	4	3.1
Candida albicans SC5314	2	1.5
Candida albicans Gu4	4	3.1
Candida albicans Gu5	128	3.1
Candida albicans DSY294	4	1.5
Candida albicans DSY296	128	1.5
Candida glabrata MTCC3814	4	1.5
Candida krusei MTCC9215	64	1.5
Candida parapsiloisis MTCC7043	2	1.5
Candida tropicalis MTCC9038	4	0.7

[a] MIC - Minimum Inhibitory Concentration of test compound that resulted in no visible growth as compared to growth control.

Results and Discussion

Synthesized cationic lipo-benzamide compounds (C2M-C18M) were evaluated for inhibition of planktonic growth of *C. albicans* strain ATCC 90028 using Broth Microdilution Assay. Four compounds from the series, C8M, C9M, C10M and C11M showed concentration-dependent growth inhibition with Minimum Inhibitory Concentration (MIC) of ≤6.2 μg ml^{-1} (Fig. 1). Compounds with an odd number of carbon chains C9M (9 carbon chain) and C11M (11 carbon chain) were found most active against ATCC 90028 with MIC value ≤ 3.1 μg ml^{-1}. Compound C9M was further tested against two pairs of azole sensitive and resistant clinical isolates (*Gu4/Gu5* and *DSY294/DSY296*) of *C. albicans* and non-*albicans* strains including *C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis.* C9M were able to inhibit the planktonic growth of all tested *Candida* strains with MIC values ranging from 0.7-3.1 μg ml^{-1} (Table 1). Compound C9M showed dramatic anti-fungal activity against both fluconazole-sensitive and resistant clinical isolates of *C. albicans*. Fluconazole (Flu) was used as standard anti-fungal agent.

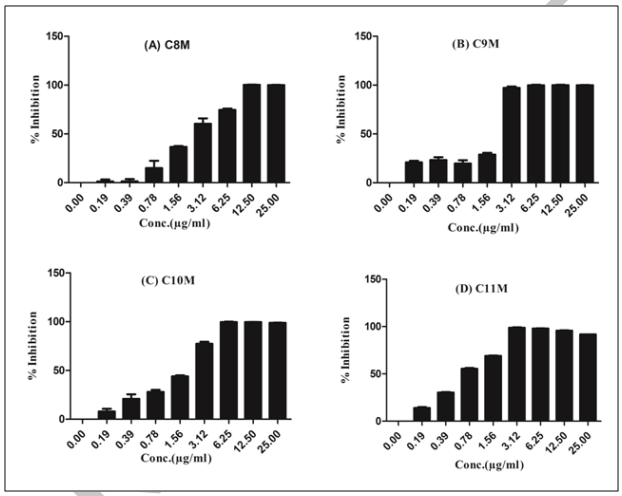


Figure 1. Antifungal activity of cationic lipo-benzamides. Inhibition of planktonic growth of *C. albicans ATCC 90028* was determined at varying concentrations of cationic lipobenzamides, (A) C8M (B) C9M (C) C10M (D) C11M. Percentage inhibition was plotted on Y-axis against the concentration of test compound on X-axis. Percentage inhibition was determined with respect to growth control in which no test compound was added.

Adhesion of *C. albicans* to polystyrene surfaces, prosthetic substrate, and host tissue leads to colonization and is an important first step leading to biofilm formation. Fungal biofilms contain population of persister cells which shows much higher resistance to anti-fungal drugs than planktonic cells. Quaternary ammonium group containing C9M was able to inhibit *Candida* cell adhesion to the polystyrene surface in a concentration dependent manner. Compound C9M inhibits 90% *Candida* cell adhesion on polystyrene surface at 12.5 μg ml^{-1} concentration compared to growth control in which no drug was added (Fig. 2A). Fluconazole was used as control anti-fungal agent for whom inhibition of cell adhesion was not observed at tested concentrations upto 25 μg ml^{-1}.

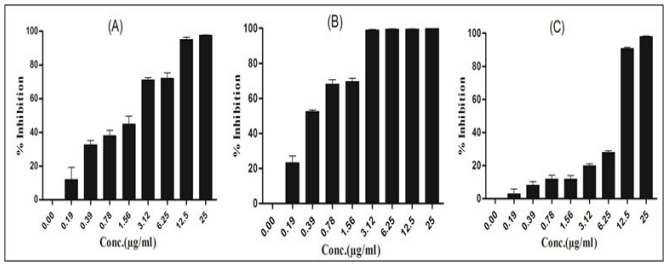


Figure 2. Compound C9M was tested for inhibition of different stages of *C. albicans (ATCC 90028)* biofilm development, (A) Adhesion (B) Formation and (C) Mature Biofilm, and inhibition was quantified using MTT metabolic assay. Inhibition of adhesion to polystyrene surface and biofilm formation was observed at 12.5μg ml^{-1} and 3.12μg ml^{-1} respectively. Mature Biofilm was inhibited at 12.5μg ml^{-1} concentration.

The deposition of organic monolayers onto solid surfaces containing quaternary ammonium groups prevents deposition and growth of bacterial biofilms [12a]. Our study reveals that C9M can

not only inhibit *Candida* biofilm formation but is also able to eradicate mature biofilms. $1x10^6$ cells of *C. albicans ATCC 90028* were allowed to grow and adhere to the polystyrene coated wells of 96 well plates. C9M was administered at different time points for identifying its role during biofilm formation and also on mature biofilm. **C9M** was able to inhibit *Candida* biofilm formation at Biofilm Inhibitory Concentration (BIC_{80}) of 3.12 µg ml^{-1}, quantified using MTT assay (Fig. 2B). Microscopic examination also revealed that biofilm formation was inhibited at different concentrations of C9M (Fig. 3). We found that C9M eradicates mature *Candida* biofilms at a Biofilm Eradicating Concentration (BEC_{80}) of 12.5 µg ml^{-1} (Fig. 2C). Fluconazole was used as a standard anti-fungal drug and no biofilm inhibition was observed at tested concentration.

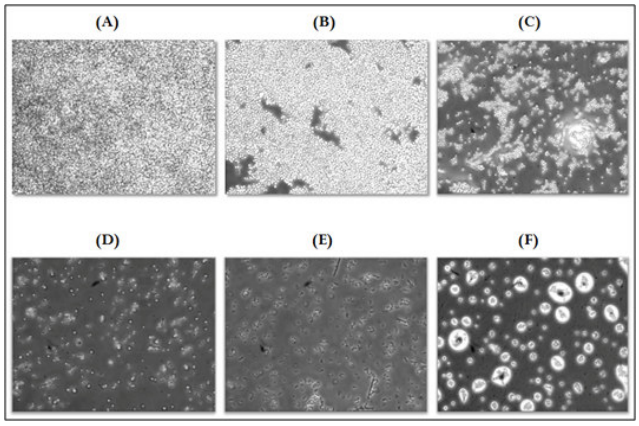


Figure 3. Qualitative analysis of inhibition of biofilm formation by C9M against *C. albicans* cells *(ATCC 90028)*. Microscopic analysis revealed inhibition of biofilm formation as compared to, (A) Growth control in which no test compound (C9M) was added. (B) to (F) Biofilm inhibition with increasing concentrations of C9M. (B) 1.56µg ml^{-1}, (C) 3.12µg ml^{-1}, (D) 6.25µg ml^{-1}, (E) 12.5µg ml^{-1} and (F) 25µg ml^{-1}, represents varying concentrations of C9M.

Cytotoxicity of **C9M** was previously evaluated in cancerous and non-cancerous cell lines. **C9M** did not exhibit cytotoxicity till 20 µmol L^{-1} (~10 µg ml^{-1}) concentration in most of the cell lines tested [14]. In the present study, we evaluated the hemolytic activity of **C9M** against human erythrocytes. We selected erythrocytes (RBCs) because of their fragile cell membrane that make them susceptible to lysis by QAC's. Hemolytic activity of **C9M** was quantified using spectrophotometer by measuring the amount of hemoglobin released upon cell lysis. The concentration of **C9M** that can lyse 90% of total RBC (HC_{90}) was determined to be 12.5 µg ml^{-1} (Fig. 4A). 1% Triton X-100 was used as a control to achieve 100% RBC lysis and buffer-treated sample was used as negative control.

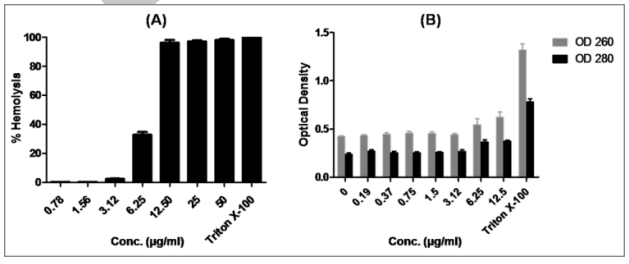


Figure 4. Cationic lipo-benzamide C9M does not lyses cells at the MIC concentration. (A) Different concentrations of C9M were incubated with RBCs. Hemoglobin released upon RBC lysis was quantified by a spectrophotometer. Percentage hemolysis was plotted on Y-axis against varying concentrations of C9M on X-axis. Complete hemolysis was observed at 12.5µg ml^{-1} concentration of C9M. (B) The release of macromolecules upon cell lysis was measured by incubating *Candida* cell with increasing concentrations of C9M. OD260 and OD280 were recorded to quantify the release of nucleic acid and proteins respectively. No significant release of macromolecules was observed till 12.5µg ml^{-1} concentration of C9M.

The ability of QAC's to inhibit the growth of microbial cells has previously been attributed to cell lysis [8]. However, in our assay, we observed that HC_{90} of cationic lipo-benzamide **C9M** (12.5 µg ml^{-1}) was 4 times higher than the MIC value (3.1 µg ml^{-1}) observed in *Candida* species. To check for *Candida* cell lysis at effective concentrations of **C9M**, we quantified the amount of macromolecules (protein and nucleic acid) released upon treatment with different concentrations of **C9M**. The increase in the amount of these macromolecules in surrounding media gives an indirect evidence of cell lysis. The release of proteins and nucleic acids was quantified spectro-photometrically by measuring OD at 280 and 260 nm respectively. In our observation, there was no increase in OD upto a concentration of 12.5 µg ml^{-1} (Fig. 4B) which is about 4 folds higher than the effective MIC value of **C9M** against *C. albicans* planktonic cells. To further verify our findings, we checked for topological alterations induced by test compound **C9M** on *C. albicans* cells using Scanning Electron Microscopy (SEM). Our observations did not reveal any significant morphological changes after **C9M** treatment when compared to the control cells (Fig. 5). Interestingly, it became evident from our study that cationic lipo-benzamide compound **C9M** does not cause lysis of *Candida* cells at a concentration that effectively inhibits planktonic growth.

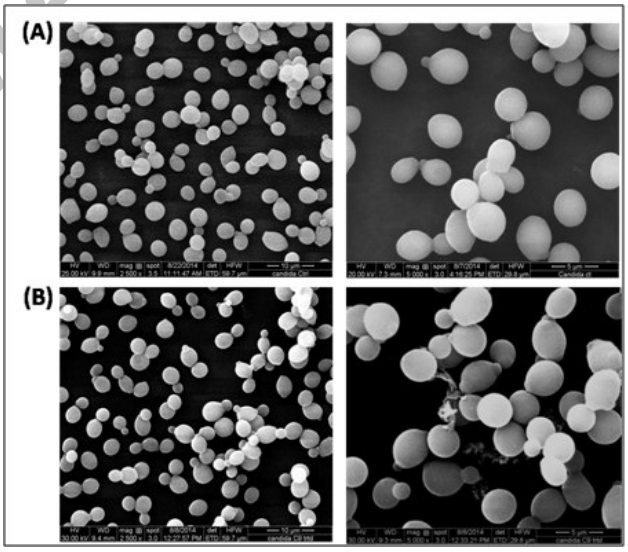


Figure 5. Representative SEM images of *C. albicans* cells *(ATCC 90028).* A) Control *C. albicans* cells at 2500X and 5000X magnification. (B) *C. albicans* cells (2500X-5000X magnification) after incubation for 24 h with 3.1 µg ml^{-1} (MIC) concentration of compound C9M.

A series of cationic lipo-benzamides containing quaternary ammonium ion were tested and found to exhibit anti-*Candida* activity. Cationic lipo-benzamides with chain lengths of 8-11 methyl units were found to inhibit the growth of *C. albicans*. The most potent among them were compounds harboring 9 and 11 carbon chain lengths. The MIC of the cationic lipo-benzamide molecule C9M was found to be quite low (0.7-3.1 µg ml^{-1}) against various fungal strains. Notably, the compound C9M was equally active against fluconazole-sensitive and resistant strains of *C. albicans* and against the tested non-*albicans* species of *Candida* as well. The chain length specificity and the anti-

microbial property have been corroborated by previous reports [15]. In one such report, Locher*et al*., have reported nostocarbolines linked to 10 and 12 methylene units were bactericidal against *Methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-resistant Enterococci (VRE),* and *E. coli* [16]. However, no specific target has been identified for QAC's till date; rather it is assumed that their effect is global. Given their chemical structures, it is possible that QACs interact with the positive charge and hydrophobic regions of microbial membranes [17]. The cationic agents are traditionally known to react with the phospholipid components in the cell membrane, thereby producing membrane distortions often leading to a complete loss of structural organization of the cells [9, 17].

C. albicans biofilm development encompasses different phases, including initial adherence, proliferation, and maturation [18]. *Candida* biofilm consists of an intricate network of yeasts, hyphae, and pseudohyphae with a subpopulation of persistor cells that are recalcitrant to antifungal therapy [19]. Due of this effect, infections associated with *Candida* biofilm represent an escalating problem in health care. The role of QAC's against both bacterial and fungal biofilms has been studied in detail in the past [20]. The ability of cationic lipo-benzamides to inhibit *Candida* biofilm has been evaluated in this study. It was found that compound C9M could not only inhibit biofilm formation but was also able to eradicate mature biofilms of *C. albicans*, a most desirable property that conventional antifungals like azoles and polyenes lack. These molecules could therefore, form the basis for the design of novel pharmacophores with potent antimicrobial activity, especially against drug-resistant isolates and *Candida* biofilms.

The fungicidal action of C9M observed by us was not associated with cell lysis since at the MIC concentration; C9M did not show any significant RBC or *Candida* cell lysis. The lack of *Candida* cell lysis was re-confirmed by SEM imaging and the quantification of macromolecules (DNA and proteins) released from *Candida* cells treated at MIC concentration of C9M. Therefore, it is possible that these molecules exhibit their fungicidal action by attaching to the cell surface and reversing the membrane charge from negative to positive in consensus with previous reports [11, 21]. However, with a narrow range of therapeutic index (ratio of MIC to hemolytic concentration of test compound), QAC's are presently restricted to topical usage and are the active ingredient of sanitizers, shampoos, mouthwash and topical anti-fungal creams. Moreover, the absence of significant *in-vitro* toxicity against mammalian cell lines and hemolysis and effective anti-fungal concentrations increases the hopes of their possible use *in-vivo*.

Conclusion

We have identified the anti-*Candida* and biofilm inhibition potential of a cationic lipo-benzamide molecule containing quaternary ammonium ion. More efforts will be necessary in order to design and test QACs or similar compounds that may be suitable for clinical usage.

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Supplementary Material

Supplementary data associated with this article can be found in the online version

Graphical Abstr

Biofilm inhibition and anti-*Candida* activity of a cationic lipo-benzamide molecule with twin-nonyl chain

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